STUDIES ON THE EFFECTS OF IRRADIATION OF CELLULAR PARTICULATES.[1] I. INHIBITION OF CYTOCHROME OXIDASE BY ULTRAVIOLET

HENRY T. YOST, JR., HOPE H. ROBSON AND IRWIN M. SPIEGLEMAN

Department of Biology, Amherst College, Amherst, Massachusetts

The effect of radiations of various types on enzyme systems has been the subject of numerous investigations. The exemplary work of Dale (1940, 1942), and of Barron and his associates (1949) has indicated that many enzymes are readily inactivated by ionizing radiation when sufficiently pure and when in dilute solution. Furthermore, their work clearly demonstrated that molecules, other than enzyme, when present in the irradiated solution, would protect the enzyme from inactivation. The current work on radiation "protection" has its origin in these findings. Such investigations have led to a clear picture of the action of ionizing radiations in the inhibition of various enzyme systems and have directed many investigators to seek the cause of gross radiation damage to organisms at the enzyme level. Similarly, much has been done to elucidate the nature of ultraviolet radiation damage to enzymes (McLaren, 1949; Lea, 1947). As early as 1897, Green had reported the destruction of diastase by ultraviolet. Nevertheless, less attention has been paid to the role of enzyme inactivation in lethal changes induced in cells by ultraviolet radiation. This is largely the result of the close correspondence of the nucleic acid absorption spectrum and most action spectra in the ultraviolet.

Although the disparity between the studies of "genetic" damage and enzyme damage is explicable in terms of the action spectra, it is surprising that in spite of the vast amount of work on the effects of radiations, little has been reported concerning their effects upon the particulate systems of cells. There are only two reports (both negative) of the effects of x-radiation on the cytochrome oxidase activity of particulates (Barron *et al.*, 1949; LeMay, 1951), and one report of the loss of the cytochromes from yeast after ultraviolet radiation (Raut, 1954). When one considers the vital role which has been assigned to particulates in the cellular metabolism, it would seem that any damage to this system would result in rather drastic changes in the cell. The remarkable similarity of the enzyme content of the particulates and the enzymatic mechanisms involving aerobic metabolism in widely differing organisms suggests a role in the continuity of the cell similar to that played by the chromosomes. For these reasons, it seemed advisable to make a study of the effects of various types of radiations on the mitochondrial fraction of the cellular particulates. The effects of "germicidal" ultraviolet radiation are reported in this paper.

For a test agent, cytochrome oxidase was chosen. This was done for three reasons: a depression in oxygen uptake has been observed by many workers following irradiation, cytochrome oxidase is closely bound to the particulate and there-

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fore clearly forms part of its structure, and the recent reports of the enhanced effect of x-radiation after the addition of CO and CN (King, Schneiderman and Sax, 1952) indicate a possible role of cytochrome oxidase in radiation damage. Furthermore, cytochrome oxidase has been shown to be the terminal oxidase of many different organisms, and it seems evident that damage to this enzyme would result in alterations in the cellular metabolism of a very basic nature.

Materials and Methods

White laboratory rats were starved overnight and sacrificed by a blow on the head. The liver was removed and placed in cold 0.85% KCl, where a good deal of the blood was washed free. The liver was weighed and pressed through a bronze

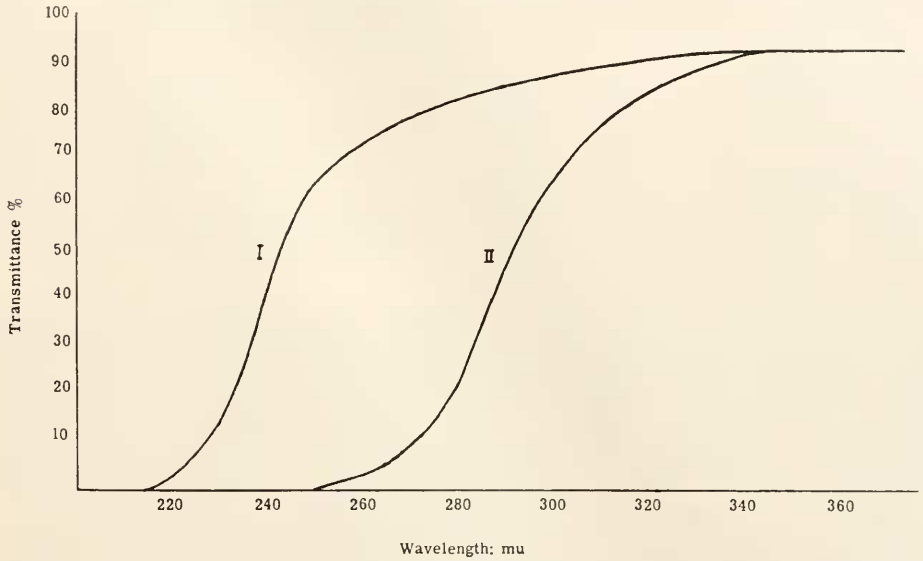

Figure 1. The transmission of the two filters used in this study. Filter I is Corning No. 7910 and Filter II is Corning No. 9700. Curves drawn from data supplied by the Corning Glass Co. and spot checked with a Beckman spectrophotometer.

sieve to remove connective tissue. The resulting mash was then suspended in cold 8.5% sucrose (50 ml.) and homogenized in a glass homogenizer with a "Teflon" pestle driven by a cone-drive stirring motor. The mitochondria were then separated from the rest of the homogenate by the method of differential centrifugation (Schneider, 1948). The mitochondrial fraction alone was kept. This fraction was washed twice with cold 8.5% sucrose, and the final sediment was suspended in 2.5 ml. sucrose solution per gram of liver. All centrifugations were carried out in a refrigerated centrifuge at 4° C.

For irradiation, a sample of the suspension was diluted 1 in 5, 1 in 10, or 1 in 15 with the same sucrose solution. (The dilutions used in each case are given with the data.) Two and five-tenths ml. were placed in a quartz flask rotated in the ultraviolet beam by a slow speed stirring motor; 2.5 ml. were kept in a Pyrex test

tube, in the cold, as a control. During the period of irradiation, the quartz flask was partially submerged in an ice-water bath maintaining a temperature under 5° C. The flask was kept 11.5 or 18.5 cm. from a G.E. germicidal lamp for all radiations. The 18.5-cm. position is referred to as the low intensity position in the rest of the paper. The intensity of the radiation was 5500 ergs/cm.^{2}/sec. at the 11.5-cm. position as measured by a G.E. light meter with ultraviolet cell. In the filter studies, different filters were placed between the source and the flask. The transmission of the two filters used is given in Figure 1. Filter I is a Corning ultraviolet transmitting filter (No. 7910) which transmits principally those wave-lengths above 2400 Å; and Filter II (Corning No. 9700) transmits principally those wave-lengths above 2800 Å. In a few experiments, the mitochondrial suspension was irradiated in an open petri plate to test the effect of the rotation in the quartz flask. When this was done, controls were also kept in the cold in an open petri plate.

In the studies done with whole cells, cells of *Parthenocissus* crown gall grown in tissue culture were used. These cells were spread out on moist filter paper in a petri dish and exposed to the ultraviolet radiation as above. The cells were then collected and the mitochondrial fraction obtained in the same manner as that used for rat liver, with the exceptions that the tissue was ground with sand in a mortar and the final suspension was made up in a total of 2 ml. sucrose. The small fraction obtained in this way showed cytochrome oxidase activity. In cases where the oxygen uptake of whole cells is reported, the results represent the endogenous respiration. All radiations of whole cells were carried out in the same way and at the same distance (11.5 cm.) from the source.

The cytochrome oxidase activity was estimated manometrically by the method of Hogeboom, Claude and Hotchkiss (1946). The main compartment of each vessel contained: 0.35 ml. of the mitochondrial suspension, 0.1 ml. Sorenson phosphate buffer (pH 7.4), 1 ml. 1.3×10^{-4} *M* cytochrome-*c* (Sigma, horse-heart) in 0.85 % NaCl, and 0.15 ml. 0.005 *M* $AlCl_3$. The center well contained 0.2 ml. 5 N KOH; and the side arm held the reducing agent, 0.15 ml. 0.228 *M* sodium ascorbate. In studies concerning the effects of added nucleotides, each flask contained 5 μM AMP (adenosine-5-phosphoric acid, free acid—Sigma), ADP (adenosine diphosphate, sodium salt—Sigma), or ATP (adenosine triphosphate, neutral sodium salt —Schwartz). In these cases, as in the case of the determination of the effect of pH on the enzyme, the buffer in the flask was changed. In the experiments with the nucleotides the buffer was increased in an attempt to keep the final pH of the reaction at 7.4. In the pH series, various phosphate buffers were used to give the desired range. In the determination of the oxygen uptake of the whole cells the vessels contained only cells and one ml. of water. The center well contained alkali as above. All determinations were made at 38° C. with the exception of the whole cells and the ones made with ATP. These two latter were done at 30° C.

Attempts to photoreactivate the system were made by placing the irradiated suspension under two 250-watt fluorescent lights for various lengths of time, as indicated in the tables. In some cases the exposure to light was made in an ice bath and in others the experiments were carried out at room temperature. The suspension was exposed to the light in a petri plate with the cover left on, and with two thicknesses of window glass between the petri plate and the source. This was done to remove any ultraviolet radiation from the fluorescent tubes.

All assays were made in triplicate. In addition, most experiments were re-

peated three or more times; some of the less important points on the dosage curve were repeated only twice.

Results

The data presented in Table I show the effectiveness of ultraviolet radiation in the inactivation of cytochrome oxidase. In this case, representative experiments are shown (chosen to correspond to the average value for several runs). It can be seen that 20 minutes of radiation are sufficient to reduce the oxidase activity to about 40% of the controls, and that at one hour only 20% of the activity is left. These values were obtained using Filter I; without the filter the source is only

Table I

The effect of ultraviolet radiation and of rotation upon cytochrome oxidase activity

(Preparation diluted 1 : 5)

Treatment	μL O_2	Per cent inactivation
Control	365.4	
20 min. UV	163.6	55.2%
Control	159.4	
60 min. UV	29.9	81.2%
Control	134.7	
60 min. rotation	124.0	7.9%

slightly more effective, the difference being due to the absorption of the filter. Table I also presents data on the effect of rotating the suspension in the quartz flask without radiation. This was done to see if there was an inactivation of the oxidase by this procedure. This particular experiment was repeated ten times, and there is an average inactivation of 8.2% with one hour rotation. This is very slight when compared to the effect of the radiation and can be ignored for most of the studies (wherever Filter I is used).

Table II presents the data obtained when different filters were used. The activity is expressed as per cent of the controls and represents an average value of all experiments. It is evident that Filter I (wave-lengths above 2400 Å) is much more effective than Filter II (wave-lengths 2800 Å). One hour of radiation with Filter

Table II

Relative effectiveness of filtered ultraviolet radiation

(Preparation diluted 1 : 5)

Filter*	Treatment	Per cent control activity
I	4 min.	80.5%
I	10 min.	63.8%
I	60 min.	19.0%
II	60 min.	81.8%
II	180 min.	62.5%

* Filter I transmits mostly wave-lengths above 2400 Å; Filter II transmits mostly above 2800 Å (see Fig. 1).

II produced about as much inactivation as 4 minutes with Filter I; three hours with Filter II is equivalent to 10 minutes with Filter I. It seems that Filter II is only about $\frac{1}{15}$ as effective as Filter I (about 6%).

Figure 2 presents the relation between the inactivation of cytochrome oxidase and the dosage of ultraviolet. The curve was obtained using Filter I. This curve is a composite and should be considered only as a rough indication of the course of inactivation. All the points represent average values from several experiments. There is some variability expected in this case, since if one uses the same rat liver for all points on the curve, the controls are slowly losing activity with age, and if one uses different preparations for the different points, there is variation in the cytochrome oxidase concentration of the initial suspensions. However, it is clear that the initial inactivation of the enzyme is rapid, but the slope of the curve becomes more gradual after 20 minutes, reaching a maximum inactivation at about

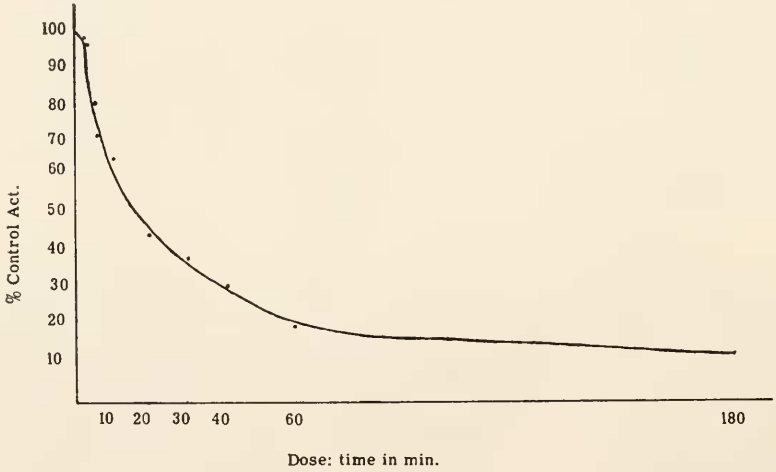

FIGURE 2. Inactivation of cytochrome oxidase by ultraviolet radiation. Radiation at high intensity; filter I; preparation diluted 1 in 5. Activity is expressed as per cent of the control activity.

one hour. This curve is similar to the inactivation curves obtained with microorganisms, and the shape is probably due to the gradual increase in protection against radiation by the inactivated enzyme. At the high intensity of radiation used in these experiments, it was not possible to determine the threshold value for the inactivation. Therefore the intensity was reduced by moving the flask away from the source. The data obtained with this lower intensity radiation are presented in Figure 3. It is evident that there is a definite threshold value for the inactivation, and that this value varies with the age of the preparation. There is distinct evidence for a slight increase in the activity of older preparations when given very low doses of ultraviolet. With higher doses (as in Figure 2) there is never any indication of an increase in activity.

Table III presents data obtained in attempts to reactivate the irradiated cytochrome oxidase. It is evident that there is neither photoreactivation nor reactiva-

tion with ADP or ATP. During the attempts to reactivate the preparation with ADP and ATP, a slight stimulation of both the controls and the irradiated preparation by the ADP and a slight inhibition by ATP was noted. It was thought that this might be an indication of the participation of these compounds in the oxidation of cytochrome-*c* as suggested recently by the work of Lehninger, Hassan and Sudduth (1954). However, it is extremely difficult to maintain a constant pH in the vessels during a test made with these compounds, and subsequent investigation indicated that the enzyme is extremely sensitive to slight variations in pH. The relationship between cytochrome oxidase activity and pH is given in Table IV. There seems to be no effect of the added ADP or ATP which is not the result of

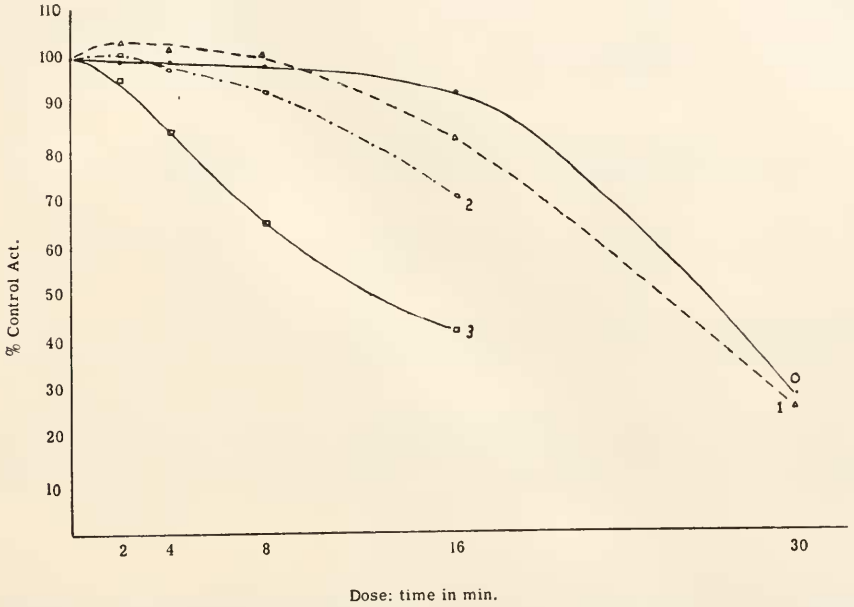

FIGURE 3. Inactivation of cytochrome oxidase preparations of varying ages by ultraviolet radiation. Source at lower intensity position; filter I; preparation diluted 1 in 15. ·———· preparation radiated same day extracted; △– – –△ preparation radiated one day later; ○—·—○ preparation radiated two days later; □——□ preparation radiated three days later.

a shift in the pH. These data seem to indicate that it is not the adenylic acid system which is being inactivated by the ultraviolet. The absence of photoreactivation in such a simple system is not surprising.

To determine the effect of ultraviolet radiation on the cytochrome oxidase activity of irradiated whole cells, cells of *Parthenocissus* crown gall were irradiated and the particulate fraction isolated after radiation. The data from these experiments are summarized in Table V. It can be seen that although 5 minutes of radiation reduces the endogenous oxygen uptake of the intact cells 17%, it takes one to two hours to achieve equivalent inactivation of the particulates removed from the irradiated cells. It would seem that the cytochrome oxidase of these cells is not greatly affected by ultraviolet radiation so long as the radiation is carried out on

TABLE III

Failure to reactivate irradiated cytochrome oxidase

(Preparation diluted 1 : 5)

Treatment	% control activity
10 min. UV	65.6%
10 min. UV + ADP	67.2%
10 min. UV	66.3%
10 min. UV + ATP	61.4%
20 min. UV	44.8%
20 min. UV + ADP	46.3%
20 min. UV	44.6%
20 min. UV + ATP	39.8%
(Preparation diluted 1 : 10)	
40 min. UV	36.7%
40 min. UV + 20 min. light*	33.2%
2 min. UV	74.4%
2 min. UV + 20 min. light†	70.0%
20 min. light†	94.2%

* Continuous light; treatment at 5° C.

† Two 10-minute light periods separated by 10 minutes of dark; treatment at room temperature.

the intact cells. Control experiments indicate no difference in the sensitivity of particulates radiated after being extracted from the cells. It is apparent that the cytochrome oxidase is protected by the rest of the cell from the radiation damage.

DISCUSSION

The data presented above indicate that cytochrome oxidase in the isolated cellular particulate is very sensitive to ultraviolet radiation. Furthermore, these data indicate that ultraviolet wave-lengths shorter than 2800 Å produce this effect. Reference to Table II indicates that Filter II is only about 6% as effective as Filter I. At these low values of inactivation, the effect of rotation of the quartz flask becomes appreciable. Table I shows that an hour of rotation decreases that activity of the preparation about 8%. Therefore, one may assume that the value of rela-

TABLE IV

The effect of pH on the activity of cytochrome oxidase

(Preparation diluted 1 : 10)

pH	Oxygen uptake (μL O_2)	% activity of 7.4
7.4	195.6	100.0
7.3	177.0	90.5
7.2	141.8	72.5
7.1	127.4	65.1
7.0	115.0	58.8

tive effectiveness given for Filter II is on the high side. From a comparison of the transmission of the two filters (Figure 1), and from a comparison of their relative effectiveness, it would seem that the effect is induced by wave-lengths in the region 2600–2700 Å. However, the source used in these studies was a germicidal lamp, and therefore most of the emitted energy was in the region of 2537 Å. One would expect a protein to be denatured by all wave-lengths below 3100 Å, with maximum effects at the absorption maxima of the protein. To test the efficiency of the source and filters on the inactivation of a protein, crystalline urease prepared by the method of Sumner (1926) and Dounce (1941) was irradiated in the same manner as the cytochrome oxidase. It was found that with Filter I, 10 minutes of radiation were necessary to produce 50% inhibition of the enzyme; to achieve similar inactivation required 60 minutes of radiation with Filter II. Therefore, it is evident that for the inactivation of a more simple protein molecule, urease, the filters have an efficiency ratio of 6. Since urease has been reported to have an absorption

TABLE V

The effect of irradiation of whole cells upon the oxygen uptake of the cells and of isolated particulates

Test	Treatment	Oxygen uptake (μL O_2)	% inactivation
Cells	Control	48.4	
	5 min. UV	40.4	17%
Cells	Control	48.3	
	120 min. UV	20.8	57%
Particulates	Control	48.1	
	60 min. UV	43.9	9%
Particulates	Control	56.6	
	60 min. UV	47.1	17%
Particulates	Control	49.2	
	120 min. UV	43.7	11%

maximum typical of simple proteins in the region of 2800 Å, and since the action spectrum for destruction of the enzyme and the absorption spectrum coincide (Kubowitz and Haas, 1933), this efficiency ratio provides us with a measure of the radiation from this source in the region 2800 Å. Since the destruction ratio for cytochrome oxidase is 15:1 with these same filters, it must be concluded that the wave-lengths below 2800 Å are about two times as efficient as those above. Therefore it seems possible to conclude that the cytochrome oxidase activity of isolated particulates is readily inactivated by wave-lengths in the region of 2600–2700 Å. Since some effect is obtained with Filter II, the wave-length cannot be lower than 2600 Å.

The fact that wave-lengths in the vicinity of the so-called "nucleic acid peak" are capable of deactivating cytochrome oxidase suggested that some nucleotide associated with the activity of the oxidase was being destroyed. Recent work by Lehninger, Hassan and Sudduth (1954) has demonstrated the formation of ATP during the oxidation of cytochrome-*c*. It was thought that perhaps either ADP or ATP was destroyed by the radiation and that this destruction was acting to inhibit

the over-all reaction. Attempts were made to reactivate the preparation by the addition of both of these compounds, without success. It is possible that there is a nucleotide bound to the enzyme, however, which is destroyed and cannot be replaced by external sources; unfortunately such an hypothesis is difficult to test. As pointed out above, in the course of these investigations it was discovered that the pH had a very great effect upon the enzyme activity, more than could be accounted for by changes in the cytochrome-*c*. It is possible that the ultraviolet acts upon the particulate in such manner that the internal pH is shifted. However, this seems unlikely when the effect remains after prolonged exposure to buffer at the optimal pH.

It might also be suggested that the ultraviolet is destroying the structure of the particulate and thus inactivating the enzyme. This seems unlikely in view of the fact that the enzyme can be solubilized and retain its activity (Eichel *et al.,* 1950). Experiments done with the solubilized preparation indicate that the oxidase is inactivated by ultraviolet radiation in the same manner as in the intact particle. This also seems to eliminate the possibility that the effect is upon some other enzyme in the oxidative pathway, whose loss inhibits cytochrome oxidase. The best assumption, at present, seems to be that the effect is upon the oxidase molecule directly. When the data presented in Figure 3 are considered, it becomes evident that at low doses there is a stimulation of oxygen uptake, under certain conditions. This effect is observed most readily in a preparation that has aged for one or two days in the cold. Most frequently this effect is associated with an increase in the activity of the controls, when compared to those of the preceding day. The simplest explanation of this "aging" effect is that the permeability of the particulate membrane is increasing with time. The effect of the ultraviolet radiation may be to hasten (or increase) this change. It should be noted that this effect is not limited to old preparations. Sometimes it is observed with fresh preparations, generally with very low doses. Apparently this effect depends upon the conditions which prevail in the cell before extraction. It will be noted that the increase with low doses of ultraviolet disappears as the preparation gets older. At this time the control activity is also dropping, indicating a loss of activity by the cytochrome oxidase, for which further shifts in permeability would not compensate. These observations may provide an explanation for the reports of stimulation of oxygen uptake by ultraviolet radiation in whole cells. In the whole cell, as can be seen in Table V, the oxidase molecule is protected by other cellular constituents. This represents an effective lowering of the dose of ultraviolet, and may, under some conditions, result in a stimulation of the activity of cytochrome oxidase. It is evident that the effect of the ultraviolet in increasing the activity of the cytochrome oxidase may not be an effect on the permeability of the particulate membrane; however, since the effect becomes more pronounced with age, it seems to be an effect on something other than the oxidase molecule itself, permeability being the most likely suspect.

It is evident that further analysis depends upon a determination of the absorption spectrum of the cytochrome oxidase molecule and an elucidation of the action spectrum for its inactivation. Neither of these problems is simple. Measurements of the absorption spectrum of a suspension of isolated particulates show no clear peaks in the ultraviolet below 2800 Å. There is a general peak between 2800–2900 Å, corresponding to the protein maxima, but below this the absorption tapers off gradually. The difference in the absorption between 2800 and 2600 Å is rather small, indicating general absorption in the ultraviolet. Attempts were made to con-

centrate the cytochrome oxidase in its solubilized form by the method of Wainio *et al.*, (1948). Isolation of the enzyme resulted in a clear (yellow) preparation having reasonable activity. However, the preparation is completely opaque to wavelengths below 2700 Å, largely the result of the extractant, desoxycholic acid. Removal of the extractant results in the precipitation of the enzyme in an insoluble form. Further work in this direction awaits a new method for the purification of cytochrome oxidase. The only indication that the molecule has an absorption peak at 2600–2700 Å is the CO spectrum obtained by Warburg and Negelein (1929). They obtained a strong peak in this region. Measurements of the action spectrum for the inactivation of the enzyme are complicated by the fact that the intensity of most monochrometers is too low to give meaningful results. The radiation time becomes excessive, and the preparation is destroyed by other factors than the radiation. A sufficiently intense monochrometer is not available in this laboratory at the present time.

The fact that ultraviolet radiation in the region 2600–2700 Å inhibits cytochrome oxidase activity suggests that one of the causes of cell death may be the destruction of this enzyme. It is well known that ultraviolet radiation in relatively low doses blocks the cell division mechanism, and at higher doses causes the death of cells. This effect has been attributed to a change in the nucleic acids of the cells. It seems quite possible that the effect is on the enzyme systems of the cells. Inactivation of one of the major terminal oxidases would undoubtedly disturb the metabolism of the cell and possibly result in death. Attempts were made to test this idea using cells of *Parthenocissus* crown gall grown in tissue culture. These cells were chosen because they can be grown in large quantities, and they lack the pigments which would interfere with the absorption of the ultraviolet. Attempts were made to study this problem with rat liver cells, but even with repeated washing of the cells they could not be totally freed of blood. The plant cells seemed to be ideal. The cell division mechanism is inhibited with relatively low doses of ultraviolet (two minutes), and the respiration is also inhibited. However, as can be seen in Table V, these effects cannot be attributed to an effect upon the cytochrome oxidase. It would appear that the effect of the ultraviolet is upon some step preceding the terminal oxidase or upon some other enzyme acting as a terminal oxidase in these cells. As cytochrome oxidase may not be the major terminal oxidase, the test is inconclusive. Tissue which is normally exposed to ultraviolet radiation and which is sufficiently clear to permit penetration (skin, cornea, etc.) is difficult to come by in sufficient quantities to extract the particulates. At the present time no final conclusion can be reached on this point. It does seem probable that exposed tissues lose the activity of cytochrome oxidase in cells in which this is a major oxidase. The consequence of this loss may be drastic or slight depending upon the degree of destruction of the enzyme and the other mechanisms present in the cell to carry on the oxidative metabolism.

Summary

Data are presented which indicate that ultraviolet radiation of 2600–2700 Å is effective in the inactivation of cytochrome oxidase in intact cellular particulates. The effect seems to be upon the oxidase molecule itself. It is suggested that this could be one cause of cell death from ultraviolet radiation.

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